PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:

Application No.:	10/517,322	Examiner:	M.J. Yu
Filing Date:	December 15, 2005	Art Unit:	1641
First Inventor:	Per MÅNSSON et al.	Customer No.:	23364
Attorney No.:	MANS3010/REF/LES	Confirm. No.:	3648
For:	COATED METAL SURFACE ON SOLID SUPPORT USEFUL IN ANALYTE DETECTION BY DISPLACEMENT		

APPEAL BRIEF UNDER 37 C.F.R.§41.37

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

MAY IT PLEASE YOUR HONORS:

This Appeal Brief is in response to the final rejection mailed on March 12, 2010. A Notice of Appeal was filed in the U.S. Patent and Trademark Office on May 18, 2010. The Appeal Brief is timely filed. The required appeal fee set forth in § 41.20(b)(2) of $270 is also submitted herewith. Any additional fees necessary for this appeal may be charged to Deposit Account No. 02-0200.

41.37(c)(1)(i) REAL PARTY IN INTEREST

The real party in interest is the Assignee of record, BIOSENSOR APPLICATIONS SWEDEN AB., SUNDBYBERG, SWEDEN.

41.37(c)(1)(ii) RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal known to appellant, appellant's legal representative or assignee.

41.37(c)(1)(iii) STATUS OF THE CLAIMS

This application contains claims 1-18. Of these claims, claims 7-11 have been cancelled or withdrawn without prejudice or disclaimer and are no longer pending. Claims 1-6 and 12-18 are pending, are under final rejection, and are the claims on appeal.

41.37(c)(1)(iv) STATUS OF AMENDMENTS AFTER REJECTION

We filed a Supplemental Amendment After Final Rejection on May 18, 2010, to correct spelling errors in claims 2 and 17. The amendment filed on May 18, 2010, was acknowledged in the Advisory Action dated May 27, 2010. An additional Supplemental Amendment After Final Rejection is being filed concurrently herewith in order to correct a spelling error in claim 5.

41.37(c)(1)(v) SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 is directed to a coated metal surface on a solid support, wherein the coating consists of a protein layer firmly attached to the metal surface, and said protein layer is coupled to linker molecules that are bound to low molecular weight antigens, wherein the linker molecules are coupled to the protein layer and are bound to the antigen via functional end groups and contain between the functional end groups an aliphatic hydrocarbon chain of 1, 2 or 3 carbon atoms, and wherein the antigens are reversibly bound to antibodies specific for the antigens (application as filed at page 2, line 29 to page 3, line 2).

Independent claim 12 is directed to a coated metal surface on a solid support, wherein the coating consists of a protein layer firmly attached to the metal surface, wherein the metal is selected from the group consisting of gold, silver, aluminum, nickel, chrome chromium and

titanium, and said protein layer is coupled to linker molecules that are bound to low molecular weight antigens, wherein the linker molecules are coupled to the protein layer and are bound to the antigen via functional end groups and contain between the functional end groups an aliphatic hydrocarbon chain of 1, 2 or 3 carbon atoms, and wherein the antigens are reversibly bound to antibodies specific for the antigens (application as filed at page 2, line 29 to page 3, line 2; and at page 3, lines 30-32 (metal surface selected from gold, silver, aluminum, nickel, chromium and titanium)).

41.37(c)(1)(vi) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Whether claims 1-3, 5, 6, 12, 13, and 15-18 are obvious under 35 U.S.C. § 103(a) as being unpatentable over Miura et al. (U.S. Patent Application Publication No. 2002/0009812) in view of Jacobs et al. (U.S. Patent No. 6,905,816) and Johnson (U.S. Patent No. 5,631,172).

II. Whether claims 4 and 14 are obvious under 35 U.S.C. § 103(a) as being unpatentable over Miura et al. in view of Jacobs et al. (U.S. Patent No. 6,905,816), Johnson, and Hauser et al. (U.S. Patent Application No. 2003/0162987).

41.37(c)(1)(vii) ARGUMENT

I. Claims 1-3, 5, 6, 12, 13, and 15-18 are not obvious over Miura et al. in view of Jacobs et al. and Johnson.

In the coated metal surface recited in the present claims, the linker molecules contain an aliphatic hydrocarbon chain of 1, 2 or 3 carbon atoms, and the antigens are reversibly bound to the antibodies. The present coated metal surface is suitable for use in an immunological displacement reaction. In contrast to the present claims, Miura et al. describes a device having linkers greater than three carbon atoms, and the antigens are not reversibly bound to the antibodies. The Miura et al. device is suitable for use in an immunological competition reaction.

In order to more clearly describe the differences between the present claims and the prior art, Appellants provide the following simplified explanations of the relevant reaction mechanisms:

In a displacement reaction, a solid support has a protein layer onto which linker molecules are coupled. The linker molecules are coupled to low molecular weight antigens, which in turn are reversibly bound to antibodies specific for the antigens (a "charged" solid support). In step 1, the mass (or the weight) of the surface layer is registered. Next, a fluid sample possibly containing the low molecular weight antigens is brought into contact with the "charged" solid support. If the soluble low molecular weight antigens (analyte antigens) of the fluid sample have a stronger affinity for the antibodies than those reversibly bound to the immobilized linker-coupled antigens, then the antibodies are displaced from their attachment to the immobilized linker-coupled antigens and form a soluble complex with the analyte antigens. Thus, some of the antibodies which were originally reversibly bound to the immobilized antigens are detached/displaced from the surface. Now the mass (or the weight) of the surface layer is registered again. If the mass (or the weight) of the surface layer has decreased, the fluid sample contained the analyte antigen.

A weight gain reaction involves an antibody-coated solid support, which, for example, could be a solid support that has a protein layer onto which some kind of linker molecules could be coupled. The linker molecules could also be coupled to antibodies specific for the analyte/antigens. In step 1, the mass (or the weight) of the surface layer is registered to obtain a base-line measurement. In step 2, a fluid sample possibly containing analyte antigens is brought into contact with the antibody coated solid support, and the analyte antigens are bound to the surface antibodies as surface-bound immunocomplexes. In order to determine whether analyte antigens are present in the sample, the mass (or the weight) of the surface layer is registered again. An increase in the mass (or the weight) of the surface layer indicates that the fluid sample contained the analyte antigen.

A competition reaction involves an antigen-coated solid support, which, for example, could be a solid support that has a protein layer onto which some kind of linker molecules could be coupled. The linker molecules could also be coupled to antigens. In step 1, a solution comprising a known amount of antibodies specific for the antigens is brought into contact with the antigen-coated solid support, resulting in the formation of surface-bound antigen/antibody complexes. Now the mass (or the weight) of the surface layer is measured. After the initial measurement, the solid support is cleared of antibodies so that the original support with surface-

bound antigens remains (step 2). In step 3, the same known amount of antibodies as used in step 1 is mixed with a fluid sample possibly containing such antigens, and antibody/antigen complexes are formed in solution if the antigens are present in the fluid sample. In step 4, the reacted fluid sample containing a mix of neutralized antigen/antibody complexes and active antibodies is then brought into contact with the solid support cleared from antibodies. Only the active antibodies in the fluid sample, which have not participated in the complex formation, will bind to the surface-bound antigens on the solid support. Now the mass (or the weight) of the surface layer is registered. Since the same amount of antibodies have been used in step 1 and stage 3, the registered mass (or weight) results in step 1 and step 4 should be the same where the fluid sample does not contain the antigen. However, if the mass (or weight) measurement in step 4 is lower than in step 1, then the antigens were present in the fluid sample since the strong binding of the antigen to the antibody forms a strong non-bound complex which will not bind to the solid support of step 1.

It should be understood that in an immunological competition reaction (as in the commonly used competition ELISA assay) there is no actual competition at the reaction surface. The fluid sample is pretreated with a known amount of specific antibodies so that soluble analyte antigens can form soluble complexes with some of the specific antibodies. The residual amount of the specific antibodies in the fluid sample is thereafter determined by complex formation with surface-bound antigens. For this competition reaction mechanism to be successful, the specific antibody should have a strong affinity for both the analyte antigens and the immobilized antigens.

As noted above, the present application is directed to a coated metal surface for analyte detection by displacement. Accordingly, the present claims recite antigens reversibly bound to antibodies specific for the antigens. In other words, the coated surface recited in the present claims is suitable for use in a displacement reaction where the affinity of the antibody to the antigen that is bound on the coated surface must be lower than the affinity to the antigen. In clear contrast to the present claims, Miura et al. is directed to competition assays, and the antigens are not reversibly bound to the antibodies as defined in the present claims.

In particular, in the competition reaction as described in Miura et al. the antibodies (in sample A) are first introduced to the antigens on the solid surface in order to detect a reference change of resonance angle (first detection). Then the antibodies are "washed away" from the surface by introducing a solution of glycine hydrochloric acid (strong acid, pH 2) into the flow cell which dissociates the specific coupling between the antibodies and the antigens on the sensor surface so that the value of the resonance angle returns to the value before the introduction of sample A into the flow cell (see, e.g., paragraph 85 of the reference). Subsequently, sample B containing a mixture of the antibodies and the antigens to be detected is introduced into the flow cell and a second resonance angle is measured (see, e.g., paragraph 86; see also, e.g., paragraph 89 where the competition reaction is described).

Appellants note the competition reaction as described in Miura et al. shows that the sample antigen and the antibody coexist in sample B, and when sample B is reacted with the conjugated antigen on the surface, the two antigens compete for reaction with the same antibodies. No antibodies leave the conjugated antigens so no reversible binding of antibody to conjugated antigen is required or desirable. A person of ordinary skill in the art would have readily recognized that in Miura et al. the antibody is not reversibly bound to the antigen bound on the surface of the sensor as is the case according to the present invention as defined in claim 1. In fact, the reference antibody must be dissociated from the surface by the use of a strong acid.

The present inventors have discovered that if the linker group is longer than 3 carbons, the affinity of the bound antigen to the antibody is too high, thus limiting displacement and decreasing the sensitivity of the assay (see, e.g., page 3, lines 20-25; page 8, lines 26-30; and page 9, lines 3-5 of the specification). In contrast to the present claims, Miura et al. describes linkers having more than 3 carbons. As evidenced by Sakai et al. (Sensors and Actuators B 49 (1998) 5-12), antibodies cannot be displaced from morphine bound to a linker with 4 carbon atoms, even when a large amount of excess unbound morphine is added (see Figure 4 at page 7 of Sakai et al.).

Appellants submit that Miura et al. does not teach or suggest that the particular linkers recited in the present claims would provide antigens reversibly bound to antibodies resulting in a coated surface suitable for analyte detection by displacement.

Furthermore, the additional references do not remedy the serious deficiencies of Miura et al. In particular, Jacobs et al. describes a linker with functional groups but does not teach or suggest that the linker can be an aliphatic hydrocarbon chain, much less an aliphatic hydrocarbon chain of a certain length. Furthermore, although Johnson describes hydrocarbon chain linkers, this reference does not teach or suggest that such linkers would be useful for coated metal surfaces, and in particular does not teach or suggest that linkers with less than 4 carbon atoms would be particularly useful in coated metal surfaces suitable for displacement assays.

In view of the above, Appellants respectfully request reversal of this rejection.

II. Claims 4 and 14 are not obvious over Miura et al. in view of Jacobs et al., Johnson, and Hauser et al.

The teachings of the Hauser et al. reference do not overcome the deficiencies in the primary references as discussed above. Accordingly, Appellants respectfully request reversal of this rejection.

CONCLUSION

In view of the above arguments, all of the rejections of the claims on appeal should be reversed. The application should be passed to issue. Such action is respectfully requested.

BACON & THOMAS, PLLC
625 Slaters Lane, Fourth Floor
Alexandria, Virginia 22314-1176
Phone: (703) 683-0500
Facsimile: (703) 683-1080

Date: June 29, 2010

Respectfully submitted,

Lisa E. Stahl, Ph.D.
Attorney for Appellants
Registration No. 56,704

41.37(c)(1)(viii) CLAIMS APPENDIX

1. A coated metal surface on a solid support, wherein the coating consists of a protein layer firmly attached to the metal surface, and said protein layer is coupled to linker molecules that are bound to low molecular weight antigens, wherein the linker molecules are coupled to the protein layer and are bound to the antigen via functional end groups and contain between the functional end groups an aliphatic hydrocarbon chain of 1, 2 or 3 carbon atoms, and wherein the antigens are reversibly bound to antibodies specific for the antigens.

2. The coated metal surface on a solid support according to claim 1, wherein the metal is selected from the group consisting of gold, silver, aluminum, nickel, chromium and titanium.

3. The coated metal surface on a solid support according to claim 1, wherein the antigens are the same or different and are bound to the same protein layer or to different patches of protein layers and are selected from the group consisting of optionally derivatized explosives and narcotics.

4. The coated metal surface on a solid support according to claim 3, wherein the explosives are selected from the group consisting of trinitrotoluene (TNT), dinitrotoluene (DNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7- tetranitro-1,3,5,7-tetrazine (HMX), pentaerythritol tetranitrate (PETN), and nitroglycerine (NG).

5. The coated metal surface on a solid support according to claim 3, wherein the narcotics are selected from the group consisting of cocaine, heroin, amphetamine, methamphetamine, cannabiols, tetrahydrocannabiols (THC), and methylenedioxy-N-methylamphetamine (Ecstacy).

6. The coated metal surface on a solid support according to claim 1, wherein the solid support is a piezoelectric crystal electrode or a glass plate or prism.

12. A coated metal surface on a solid support, wherein the coating consists of a protein layer firmly attached to the metal surface, wherein the metal is selected from the group consisting of gold, silver, aluminum, nickel, chromium and titanium, and said protein layer is coupled to linker molecules that are bound to low molecular weight antigens, wherein the linker molecules are coupled to the protein layer and are bound to the antigen via functional end groups and contain between the functional end groups an aliphatic hydrocarbon chain of 1, 2 or 3 carbon atoms, and wherein the antigens are reversibly bound to antibodies specific for the antigens.

13. The coated metal surface on a solid support according to claim 12, wherein the antigens are the same or different and are bound to the same protein layer or to different patches of protein layers and are selected from the group consisting of optionally derivatized explosives and narcotics.

14. The coated metal surface on a solid support according to claim 13, wherein the explosives are selected from the group consisting of trinitrotoluene (TNT), dinitrotoluene (DNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7- tetranitro-1,3,5,7-tetrazine (HMX), pentaerythritol tetranitrate (PETN), and nitroglycerine (NG).

15. The coated metal surface on a solid support according to claim 13, wherein the narcotics are selected from the group consisting of cocaine, heroine, amphetamine, methamphetamine, cannabiols, tetrahydrocannabiols (THC), and methylenedioxy-N-methylamphetamine (Ecstacy).

16. The coated metal surface on a solid support according to claim 12, wherein the solid support is a piezoelectric crystal electrode or a glass plate or prism, the antibodies are more weakly bound to the immobilized antigens than to an analyte antigen to be tested for by displacement of the antibody from the immobilized antigen.

17. The coated metal surface of clam 16, wherein the antibodies are monoclonal antibodies produced with the same immobilized antigen linked by a longer linker than the 1-3

carbon atom linker for the coating of the coated metal surface to Keyhole Limpet Hemocyanin (KLH).

18. The coated metal surface of claim 17, wherein the antibody has sub-nanomolar affinity to the antigen.

41.37(c)(1)(ix) EVIDENCE APPENDIX

Sakai et al., *A surface plasmon resonance-based immunosensor for highly sensitive detection of morphine,* Sensors and Actuators B 49 (1998) 5-12; originally submitted with the Information Disclosure Statement filed on December 30, 2008.

41.37(c)(1)(x) RELATED PROCEEDINGS APPENDIX

None.

SPR

ELSEVIER

Sensors and Actuators B 49 (1998) 5–12

SENSORS AND ACTUATORS B CHEMICAL

A surface plasmon resonance-based immunosensor for highly sensitive detection of morphine

Go Sakai [a,*], Kyoko Ogata [a], Taizo Uda [b], Norio Miura [a], Noboru Yamazoe [a]

[a] *Department of Materials Science and Technology, Graduate School of Engineering Sciences, Kyushu University, Kasuga-shi, Fukuoka 816, Japan*
[b] *School of Bioresources, Hiroshima Prefectural University, Shoubara-shi, Hiroshima 727, Japan*

Received 1 October 1997; received in revised form 9 February 1998; accepted 11 February 1998

Abstract

Highly sensitive and selective detection of morphine (MO) based on surface-plasmon-resonance (SPR) was realized by using an anti-MO monoclonal antibody and MO–bovine serum albumin (MO–BSA) conjugate (antigen). MO–BSA was immobilized on the Au thin film of the SPR sensor chip by physical adsorption. The incident angle of the SPR system using the MO–BSA immobilized chip increased almost linearly with increasing concentration of antibody up to ca. 5 μg ml^{-1} (ppm). The addition of MO to the antibody solution (5 ppm) was found to reduce the incident angle shift sharply because of the inhibition effect of MO. Based on this inhibiting principle, the present sensor could detect MO in the concentration range 0.1–10 ng ml^{-1} (ppb). The affinity constants between the antibody and the antigens (MO and MO–BSA) could be obtained by assuming a Langmuir adsorption model for the immunoreaction system.

Keywords: Morphine; SPR; Immunosensor

1. Introduction

Morphine (MO) is a useful drug in relieving patients of severe pain, but it's excessive or habitual use frequently causes toxic symptoms. In order to prevent such toxic symptoms from occurring, sensitive monitoring of MO in blood or urine is necessary. It is reported that about 90% of orally administrated MO is excreted in urine within 24 h, and that about 10% of the excreted MO remains unchanged. The MO concentration in urine would thus be less than 1 μg ml^{-1} (ppm). HPLC [1], radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and so on have been developed for this purpose. These methods, though highly sensitive, need time-consuming and/or tedious procedures so that simpler detection methods are desired. Recently a compact electrochemical sensor for MO was proposed, but its detection range 0.1–10 μg ml^{-1} (ppm) [2] is still insufficient to meet the critical concentration of MO in blood and urine, because blood or urine samples are usually diluted extensively before exposure to the sensor in order to suppress unfavorable interactions.

There have been many reports on optical assays, such as the grating coupler [3,4], reflectometric interference spectrometry [5,6], Mach–Zehnder sensing [7,8] and so on for detecting large and/or small molecules. These optical assay system have good potential for monitoring the interaction between the analyte and sensor substrate considered for high sensitivity and reliability. In the variety of optical sensing methodology, an SPR-based sensor is wide spread as a useful tool for detecting chemical species in gaseous and liquid media. There have also been several reports on SPR-based immunosensors for detecting proteins such as IgE and H-FABP [9,10], but sensitive detection of small molecules has recently received increased attention [11–14].

This situation motivated us to examine the possibility of sensitive MO monitoring by combining an immunoreaction and a surface-plasmon-resonance (SPR) phenomenon. As an example of detecting such a small molecule, we have already reported that methamphetamine (molecular weight (MW) 149) can sensitively

* Corresponding author. Tel.: +81 92 5837537; fax: +81 92 5752318.

0925-4005/98/$19.00
PII S0925-4005(98)00107-5

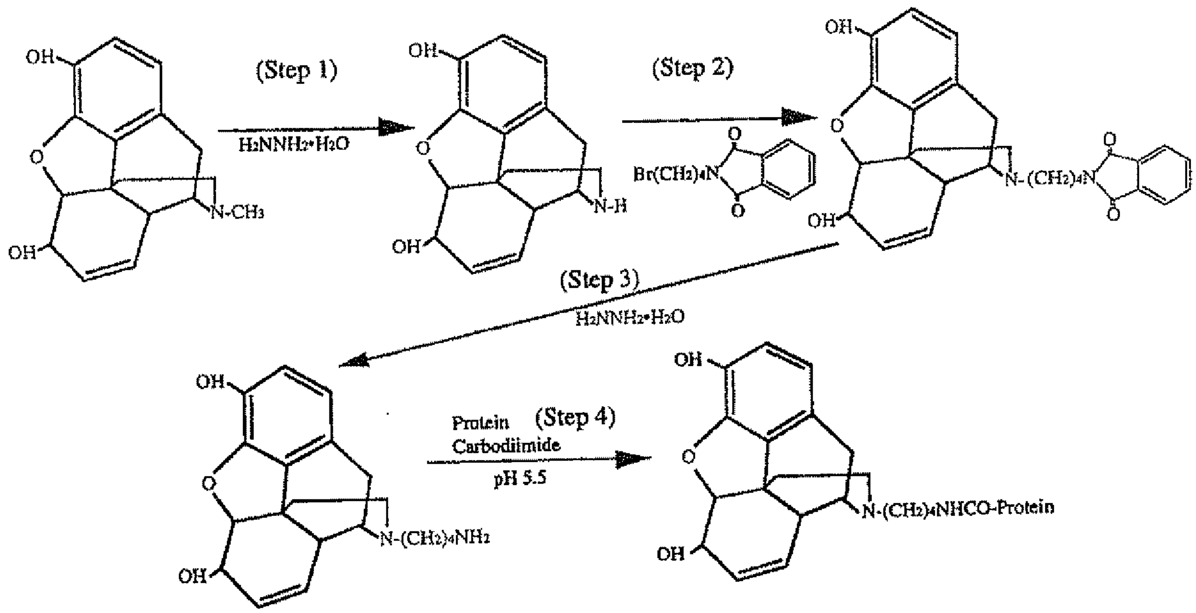

Fig. 1. Synthesis procedure for MO–BSA from morphine.

be detected based on a QCM technique coupled with an inhibitive method using a methamphetamine–protein conjugate [15]. In the present study we aimed at extending this method to a SPR-based immunosensor for MO (MW 285).

2. Experimental

MO has no immunogenicity due to its small molecular weight. So it was combined chemically with bovine serum albumin (BSA) to form a MO–BSA conjugate (MW ca. 69000), as shown in Fig. 1. MO was dissolved in a chloroform–methyl chloroformate mixed solvent at 65°C in the presence of sodium hydrogen carbonate. After vacuum distillation, the residue was mixed with hydrazine and refluxed at 115°C for 12 h to obtain normorphine (NM) (step 1). Then a DMF solution of *N*-(4-bromobutyl)phthalimide was added to the system together with sodium hydrogen carbonate and refluxed at 115°C for 2 h. The *N*-(4-phthalimidebuthyl)normorphine produced was extracted and purified to form an ethyl acetate–methanol (5:1) solution of it by means of a standard method consisting of vacuum distillation, solvent extraction and silica-gel column-assisted separation. The intermediate, thus purified, was dissolved in a mixed solution of hydrazine and allyl alcohol followed by refluxing at 115°C for 1 h under an N_2 atmosphere (step 3). The solution containing *N*-(4-aminobuthyl)normorphine produced was mixed with a DMF solution of BSA and 1-ethyl-3-(3-dimethylaminoprooyl)carbodiimide hydrochloride at room temperature (step 4) (Fig. 1). The final product (MO–BSA) was purified by means of dialysis against high purity water, before it was solidified by a freeze-drying method. All of the intermediate products were identified at the respective steps by means of mass spectrometry (MS) and nuclear magnetic resonance (NMR). An anti-MO monoclonal antibody (MW ca. 150000) was prepared by injection of the MO–BSA conjugate into mice followed by cultivation of the spleen cell of the mice. The detailed procedure is described elsewhere [16].

The sensor assembly used consisted of an SPR measuring system (SPR-20, Denki Kagaku Keiki, Japan) attached to a sensor chip, a flow-through cell (10 mm length, 3 mm width and 0.2 mm height) and a microtube pump. The angular resolution of the SPR system is $3 \times 10^{-3\circ}$. The sensor chip was prepared by depositing a 35 nm thick Au film on one side of a slide glass with a sputtering technique. Each of MO, MO–BSA, and the antibody was dissolved in phosphate-buffered saline (PBS, pH 7.2), and a fixed volume of the solution (1 cm^3) was allowed to circulate through the flow-through cell at a rate of 0.26 cm^3 min^{-1}. MO–BSA or the antibody was immobilized on the Au film of the sensor chip by physical adsorption, before exposure to MO-containing solutions. A shift in incident angle of the SPR system as a sensing signal was measured at room temperature based on the 'focused beam' principle.

3. Results and discussion

3.1. Attempt with antibody-immobilized chip

The anti-MO monoclonal antibody binds to both MO and the conjugate antigen (MO–BSA), or MO behaves as a kind of inhibitor to the immunoreaction between the antibody and MO–BSA. Therefore MO might be detected by the antibody- and the MO–BSA-immobilized sensor chips. First we tested the former chip. When a solution of the antibody (100 ppm) was allowed to flow over the sensor chip, a clear shift (ca. 0.4°) in the incident angle of the SPR spectrum was observed. This indicates that the antibody was immobilized on the surface of the Au film by physical adsorption. On subsequent exposure to an MO–BSA solution (5 ppm), a further gradual shift (0.3°) in the incident angle was observed, reflecting the immunoreaction between the immobilized antibody and MO–BSA, while no such change in the incident angle was observed on exposure to an MO solution (100 ppm) as expected (Fig. 2). The shift due to the immunoreaction was brought back to the initial value when the sensor chip was washed with glycine–HCl buffered solution (pH 2.0). It was found that the incident angle shift was almost saturated when the concentration of MO–BSA solution was 5 ppm and above, as shown in Fig. 3. However, the incident angle shift to 5 ppm MO–BSA for this device decreased gradually with an increasing number of runs. This result suggests that the immobilized anti-MO antibody is not stable enough under this condition. In addition, as shown in Fig. 4, the incident angle shifts were found to remain almost unchanged even when MO was added up to 100 ppm to the 5 ppm MO–BSA solution. This indicates that MO did not inhibit the immunoreaction between the immobilized antibody and MO–BSA under the present condition. This point will be discussed later (Section 3.5).

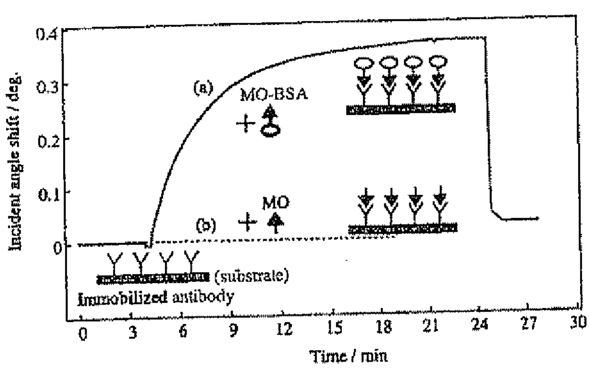

Fig. 2. Response transients of the anti-MO antibody-immobilized sensor chip to 5 ppm MO–BSA solution (a) and 100 ppm MO solution (b).

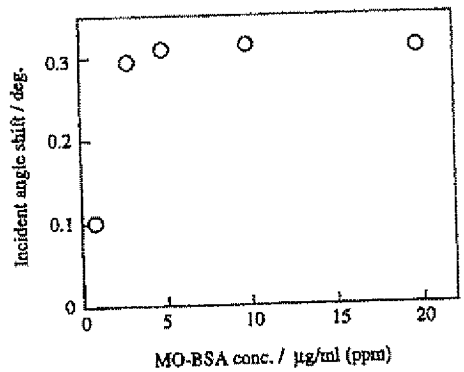

Fig. 3. The dependence of the incident angle shift of the anti-MO antibody-immobilized sensor on the MO–BSA concentration.

3.2. MO sensing principle by MO–BSA-immobilized chip

We then examined another method using the MO–BSA-immobilized sensor chip. The principle of the method is shown schematically in Fig. 5. The MO–BSA-immobilized chip increases its incident angle by $\Delta\theta$ on contact with a solution of a fixed concentration of the antibody due to the immunoreaction. When the antibody solution is mixed with a certain amount of MO, a part of antibody will react with MO to become inactive. On exposure of the sensor chip to the mixed solution, the immunoreaction takes place to a reduced extent, causing a shift in the incident angle to be attenuated from $\Delta\theta_0$ to $\Delta\theta$. If one chooses the concentration of the antibody properly, the incident angle shift ($\Delta\theta$) would be a sensitive function of the concentration of MO injected into the antibody solution.

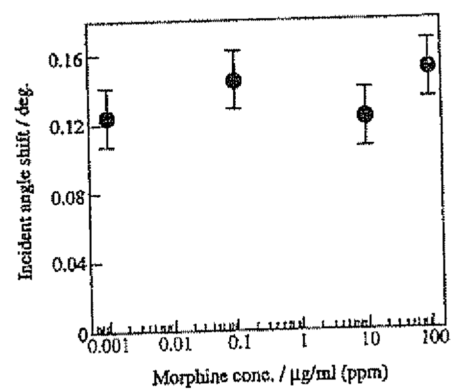

Fig. 4. The incident angle shift of the anti-MO antibody-immobilized sensor as correlated with the concentration of MO under a fixed concentration (5 ppm) of MO–BSA.

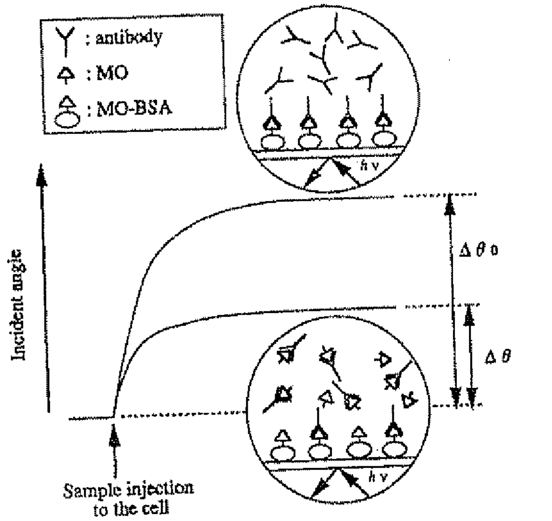

Fig. 5. The principle of MO detection by the SPR immunosensor.

3.3. Immobilization of MO–BSA

MO–BSA was immobilized on the sensor chip by physical adsorption. Fig. 6 shows the transient of the incident angle on exposure to an MO–BSA solution (100 ppm). The incident angle increased rapidly in the initial stage, followed by a more gradual increase to reach a steady state in about 15 min. The total increment of the incident angle due to the adsorption of MO–BSA was as large as ca. 0.85°. No incident angle shift was observed on the subsequent exposure to the flow of PBS solution, also shown in the figure, confirming that the immobilized MO–BSA was stable. Finally the chip was exposed to a BSA solution (1000 ppm) to block the non-specific adsorption sites of the sensor chip. This treatment resulted in an increase in the incident angle by ca. 0.12°. After this treatment, the incident angle was no longer changed by the subsequent

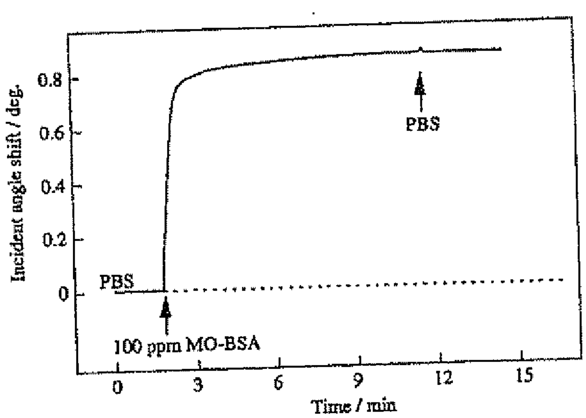

Fig. 6. Response transient of the incident angle to 100 ppm MO–BSA solution for the SPR immunosensor.

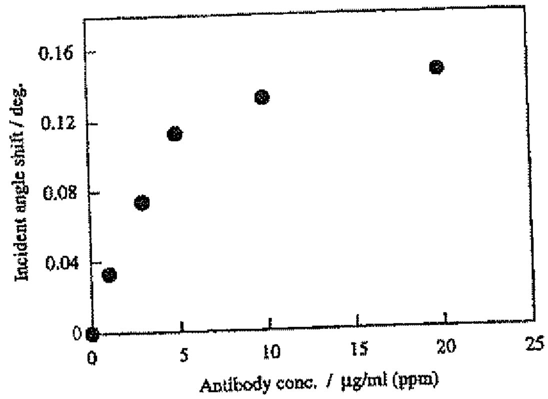

Fig. 7. The dependence of the incident angle shift of the MO–BSA-immobilized SPR sensor on the antibody concentration.

flow of 20 ppm BSA solution. The MO–BSA immobilized chip thus prepared was exposed to PBS solutions containing various concentrations of the antibody. These measurements could be carried out repeatedly, because the antibody adsorbed on the chip could be cleaned off by exposure to a glycine–HCl buffered solution (pH 2.0). Fig. 7 shows the resulting data of the incident angle shift ($\Delta\theta_0$). The incident angle shift increased noticeably with increasing concentration of the antibody up to ca. 5 ppm, and then gradually approached a saturation value (ca. 0.15°). From this behavior, the use of the 5 ppm antibody solution was judged to be best for the MO sensing experiments based on the inhibiting method.

3.4. MO sensing performances

MO was added to the monoclonal antibody solution (5 ppm) at various concentrations and then the solution was incubated for 30 min, prior to flowing over the sensor chip. Fig. 8 shows a series of response transients obtained. In the absence of MO, the incident angle shifted upward by about 0.1°. With an increase in MO concentration, the shift decreased remarkably due to the inhibition effect of MO. These measurements could be repeated about 10 times on the same sensor chip by washing off the adsorbed antibody with glycine–HCl buffered solution (pH 2.0). In further runs, the incident angle shift degraded gradually.

As shown in Fig. 9, $\Delta\theta$ was very sensitive to a change in MO concentration in the range 0.1–10 ppb. Above this range, the shift became almost nil because the whole of the antibody (5 ppm) had been inactivated with excessive amounts of MO. It can be shown that the MO:antibody molar ratio becomes unity at 10 ppb MO. It is notable that the present assay system could detect MO even at concentrations of less than 1 ppb.

It was confirmed that a similar MO sensing performance could be obtained when the PBS solution (car-

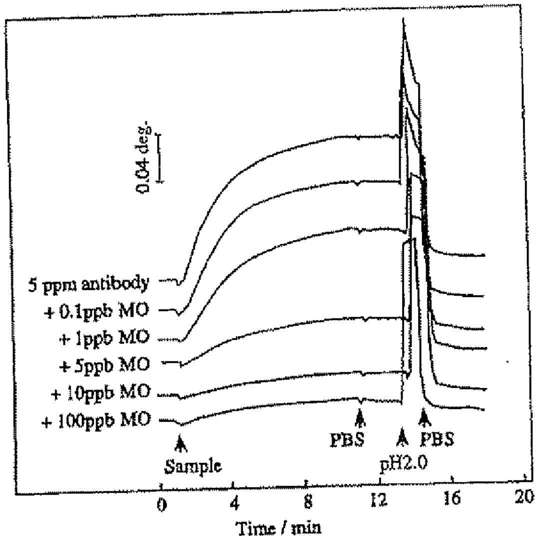

Fig. 8. Response transients of the MO–BSA-immobilized sensor to 5 ppm antibody solution containing various amounts of MO.

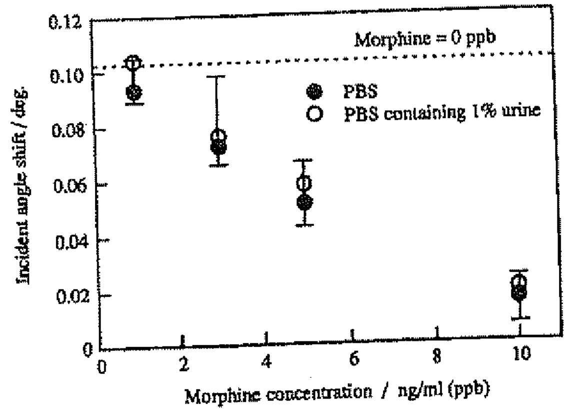

Fig. 10. Dependence of the incident angle shift of the MO–BSA-immobilized sensor to the MO concentration under a fixed concentration of antibody (5 ppm) with or without 1% urine. (The error bars of the PBS solution represent the range of data obtained with three sensor chips.)

rier solution) was mixed with 1% human urine. Fig. 10 shows the dependence of the incident angle shift on the MO concentration for the PBS solution with or without 1% urine. The incident angle shifts for the urine-containing sample solutions were slightly larger than those without urine. This means that the present system is applicable to the detection of MO in human urine in practice without serious interference by non-specific adsorption or a change in the dielectric properties. The lowest detection limit of MO seems to be ca. 2 ppb for the urine-containing samples, which corresponds to a detection limit of about 200 ppb MO for actual human urine before dilution. These excellent performances appear to show promise for the present SPR-based sensor to monitor MO in human body fluids.

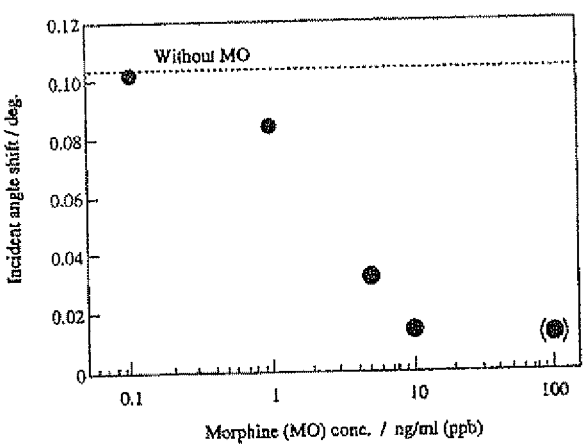

Fig. 9. The incident angle shift of the MO–BSA-immobilized SPR sensor as correlated with the concentration of MO under a fixed concentration (5 ppm) of antibody.

3.5. Evaluation of affinity constants

SPR [17,18] and a quartz crystal microbalance (QCM) techniques [19,20] have been used for in situ measurements of several immunoreactions between antibodies and antigens. However, there have been few investigations on the inhibition effects important in the present sensor. We tried to determine the affinity constants of the anti-MO antibody to MO–BSA and MO.

The immunoreaction between the anti-MO antibody (Ab) and the MO–BSA immobilized on the Au surface can be considered as the adsorption of the antibody molecules to the immobilized MO–BSA sites (*).

$$\mathrm{Ab} + * \overset{K_1}{\rightleftarrows} \mathrm{Ab}-* \tag{1}$$

Here K_1 is the affinity or adsorption constant. If Langmuir-type adsorption is assumed under the condition that the amount of Ab is far larger than that of MO–BSA (adsorption sites), the coverage (S) of the adsorption sites by the Ab molecules is given by

$$S = \frac{K_1[\mathrm{Ab}]}{1 + K_1[\mathrm{Ab}]} \tag{2}$$

where [Ab] stands for the molar concentration of Ab. It is assumed that the incident angle shift, $\Delta\theta_{0,\max}$, due to the immunoreaction is proportional to S, i.e.

$$\Delta\theta_0 = C \times S \tag{3}$$

C is a proportionality constant which should eventually be equal to the maximum incident angle shift ($\Delta\theta_{0,\max}$) at the saturated adsorption ($S = 1$). Eqs. (2) and (3) give rise to

$$\frac{[\mathrm{Ab}]}{\Delta\theta_0}=\frac{[\mathrm{Ab}]}{\Delta\theta_{0,\max}}+\frac{1}{\Delta\theta_{0,\max}\times K_1} \quad (4)$$

$[\mathrm{Ab}]/\Delta\theta_0$ should be linear to [Ab], the slope and intercept giving the values of $\Delta\theta_{0,\max}$ and K_1, respectively. The data in Fig. 7 are replotted in this way in Fig. 11(a). A linear correlation was obtained as indicated, which gave $\Delta\theta_{0,\max}=0.174°$ and $K_1=4.13\times10^7\ \mathrm{M}^{-1}$.

When MO is added to the system, it combines with the antibody with affinity constant K_2.

$$\mathrm{Ab}+\mathrm{MO}\overset{K_2}{\rightleftarrows}\mathrm{Ab}-\mathrm{MO} \quad (5)$$

The concentration of free antibody molecules is given by

$$[\mathrm{Ab}]=\frac{[\mathrm{Ab}]}{1+K_2[\mathrm{MO}]} \quad (6)$$

where $[\mathrm{Ab}]_0$ is the initial concentration of Ab. By inserting Eq. (6) into Eq. (4) and rearranging, one obtains the following equation.

$$\frac{1}{\Delta\theta}=\frac{1}{\Delta\theta_0}+\frac{K_2}{\Delta\theta_{0,\max}K_1[Ab]_0}[MO] \quad (7)$$

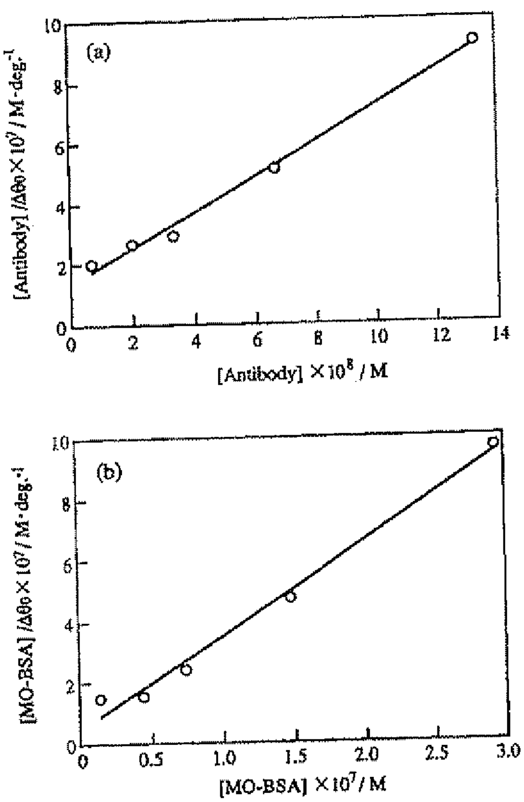

Fig. 11. The relationships between reactant concentration and reactant concentration/$\Delta\theta 0$. (a), MO–BSA immobilized sensor chip; (b), antibody immobilized sensor chip.

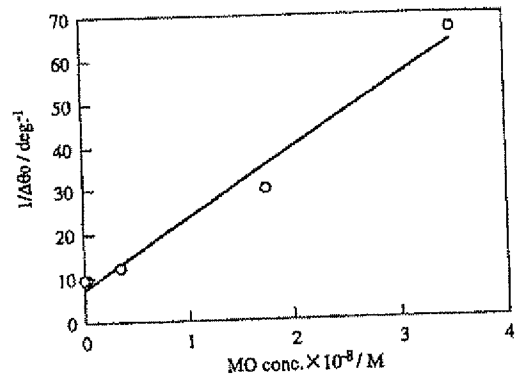

Fig. 12. The relationship between $1/\Delta\theta_0$ and [MO].

Here $\Delta\theta$ and $\Delta\theta_0$ are the angle shifts in the presence and absence of MO, respectively. The inverse $\Delta\theta$ should thus be linear to [MO], K_2 being got from the slope and the known values of $\Delta\theta_{0,\max}$, K_1 and $[\mathrm{Ab}]_0$. The data for the MO concentration range up to 10 ppb in Fig. 9 are replotted in Fig. 12. The linear correlation obtained gave $K_2=3.84\times10^8\ \mathrm{M}^{-1}$. This value of K_2 is about 5 times as large as K_1 estimated previously, and thus appears to be reasonable in accounting for the fairly strong inhibition effect of MO on the immunoreaction.

The same discussion should be applicable to the case where MO–BSA was allowed to react with the immobilized antibody (Section 3.1); [Ab] in Eq. (4) is now replaced by the molar concentration of MO–BSA. As analyzed in the same way, the data in Fig. 3 in fact give the linear correlation shown in Fig. 11(b), allowing us to estimate $\Delta\theta_{0,\max}=0.326°$ and $K_1'=6.93\times10^7\ \mathrm{M}^{-1}$. The value of K_1' was fairly close to the K_1 value estimated previously. This suggests that the immunoreaction should have taken place almost irrespective of whether MO–BSA or the antibody was immobilized. Nevertheless, the inhibition effect by MO could not take place when the antibody was immobilized. The reason for such a difference in inhibition effect is not clear. It has been reported that affinity constants of proteins are usually reduced, often by as much as two orders of magnitude, when the proteins are immobilized on a solid surface. It is possible that the affinity constant (K_2') between the immobilized antibody and MO is far smaller than that (K_2) between the free antibody and MO. If this is the case, MO would not be able to inhibit the immunoreaction of the immobilized antibody with MO–BSA as observed. However, this will have to be checked by further investigation.

4. Conclusions

An SPR-based sensor very sensitive to morphine (MO) was made by using an MO–BSA conjugate (antigen) immobilized on the sensor chip and an anti-MO monoclonal antibody. The addition of MO to the antibody solution (5 ppm) reduced the incident angle shift sharply due to the inhibition effect of MO. Based on this inhibiting principle, the present sensor could detect MO in the concentration range 0.1–10 ng ml^{-1} (ppb). The affinity constants of the antibody and the antigens (MO or MO–BSA) could be evaluated based on a simple adsorption model. The high MO sensitivity was accounted for by a large affinity of the antibody to MO.

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Biographies

Go Sakai has been a research associate at Kyushu University since 1996. He received a BEng degree in applied chemistry in 1991 and a doctorate in engineering in 1996 from Kyushu University. His current research work is focused on the development of chemical sensors as well as functional inorganic materials.

Kyoko Ogata received a BEng degree in biochemical engineering and science in 1995 from the Kyushu Institute of Technology and an MEng degree in materials science and technology in 1997 from Kyushu University. She currently works at the NEC.

Taizo Uda has been a professor at Hiroshima Prefectural University since 1994. He received the BEng degree in 1969 from Yamaguchi University and a doctorate in engineering in 1975 from Kyushu University. His current research concentrates on the basic research of immunological chemistry and its application to biosensors.

Norio Miura has been an associate professor at Kyushu University since 1982. He received the BEng degree in applied chemistry in 1973, his MEng degree in 1975 from Hiroshima University and a doctorate in engineering in 1980 from Kyushu University. His current research concentrates on the development of

new chemical sensors as well as other electrochemical functional devices such as ECD and secondary batteries.

Noboru Yamazoe has been a professor at Kyushu University since 1981. He received the BEng degree in applied chemistry in 1963 and a doctorate in engineering in 1969 from Kyushu University. His current research interests include the development and application of functional inorganic materials.